DESCRIPTION

NOVEL ACTIN-RELATED CYTOSKELETAL PROTEIN "LACS"

Technical Field

The present invention relates to novel actin-related cytoskeletal proteins, and genes encoding the proteins. Furthermore, the present invention relates to inventions utilizing the proteins and genes of this invention, such as pharmaceuticals comprising the proteins or genes as active ingredients.

Background Art

Nitric oxide (NO) (Moncada and Higgs, Eur. J. Clin. Invest. 21 (4): 361-74 (1991)) is a messenger molecule that takes on various physiological roles in the cardiovascular, nervous, and immune systems (Griffith *et al.*, J. Am. Coll. Cardiol 12: 797-806 (1998)). NO is produced together with L-citrulline from vascular endothelial cells, using arginine as a substrate and two types of nitric oxide synthases (NOSs; cNOS (constitutive) and iNOS (inductive); Bredt and Snyder, Proc. Natl. Acad. Sci. USA 87: 682-5 (1990); Janssens *et al.*, J. Biol. Chem. 267: 22964 (1992); Lyons *et al.*, J. Biol. Chem. 267: 6370-4 (1992)). Reports show that NO is involved in: (1) vasodilation mediated by vascular endothelial cells (Tanner *et al.*, Circulation 83: 2012-20 (1991)); (2) inhibition of vascular intimal thickening (Garg and Hassid, J. Clin. Invest. 83: 1774-7 (1989)); (3) mediation of vasodilation in nonadrenergic noncholinergic nerves; (4) nerve cell death; (5) action as a neurotransmitter; (6) long-term potentiation and long-term depression of memory; (7) bactericidal effect of macrophages and neutrophils; (8) release of insulin from pancreatic β-cells (Life Science 49: L213-7 (1991)); (9) carcinogenesis (Gastoloenterology 103: 1260-6 (1992)); (10) antiplatelet effect (Radomski *et al.*, Proc. Natl. Acad. Sci. USA 87: 5193-7 (1990)); and such. NO also has various antiarteriosclerotic and cardioprotective functions in the cardiovascular system. Thus, administration of NO synthase inhibitors causes cardiovascular remodeling such as inflammatory and proliferative changes in the cardiovascular tissues, thickening of the tunica media, perivascular fibrosis, and cardiomegaly.

L-NAME (N^G-Nitro-L-arginine methyl ester, hydrochloride) is a widely used NO synthase inhibitor that inhibits cNOS and iNOS. Continuous administration of L-NAME to rats can produce rats with inhibited NO production. In such model rats, increase of blood pressure as well as cardiovascular inflammatory and proliferative changes (infiltration of monocytes/macrophages, increase of MCP-1, elevation of NF-κB activity, etc.) occur within one week of L-NAME administration, and cardiovascular remodeling is observed from the

fourth week onwards. Eventually, the rats die due to cardiac failure, renal failure, cerebral infarction, or such. Inflammatory and proliferative changes and arteriosclerotic lesions in rats with inhibited NO production are known to disappear when the effects of angiotensin II (AngII) or MCP-1 are suppressed.

Rho is a low-molecular-weight G protein that regulates the adhesion of cells to the extracellular matrix and vascular endothelium, and is involved in various processes including cell-substrate adhesion, cell migration, neurite retraction, cytokinesis, and cell cycle progression from G_1 to S phase. Many of these effects are due to the rearrangement of the actin cytoskeleton. The actin cytoskeleton is modulated by using Rho-regulated adhesion as a supporting point, and it enables the migration of cells into tissues and passing of cells through intercellular space. Rho is inactive in the GDP-bound form, and becomes active upon GTP binding. The activated GTP-bound Rho acts on effector molecules that are further downstream in the pathway. Rho-associated kinase (Rho-associated coiled-coil-forming protein kinase; ROCK) is a protein kinase and one of the Rho downstream effectors. Rho induction of the actin cytoskeleton occurs at different locations in the cell cycle to produce different skeletons of specific forms.

ROCK is a serine/threonine kinase having a molecular weight of 160 kDa. It has a kinase domain at the N terminus, a coiled-coil-forming region in the middle, and a membrane-bound domain at the C terminus. Previous analyses have shown that ROCK regulates the actin skeleton through a number of pathways (M. Maekawa *et al.*, Science 285: 895-8 (1999)). In one of the pathways, myosin phosphatase is inactivated, and myosin is activated by directly phosphorylating the myosin light chain to induce actomyosin contraction. Another pathway involves the activation of LIM kinase. Activated LIM kinase becomes inactive upon phosphorylation of the actin-binding protein cofilin. As a result, the actin depolymerization activity of cofilin is suppressed, increasing filamentous actin. Yet another pathway involves phosphoactivation of Na^+/H^+ exchanger isoform-1. Upon activation, the exchanger promotes binding of the ERM (Ezrin/Radixin/Moesin) protein, and induces the binding of actin to cell membrane. ROCK is considered to contribute to the formation of cell membrane-bound actomyosin bundles through such pathways.

Disclosure of the Invention

The present inventors have reported that NO-mediated changes in cardiovascular remodeling can occur due to a local increase of angiotensin convertase (ACE) activity in cardiac tissues, and can be suppressed almost completely by ACE inhibitors and angiotensin II receptor (AT1R) antagonists. However, many facts still remain unclear such as the mechanism of local activation of the renin-angiotensin system (RAS), the mechanism involved

in the changes of cardiovascular architecture following signaling, etc. Thus, identification of genes that play important roles in the development of cardiovascular lesions is desired. Such genes and proteins encoded by these genes are also considered to be important in terms of the prevention and treatment of cardiac diseases.

The present inventors aimed to isolate and identify novel genes with important roles in the development of cardiovascular lesions. Therefore, the inventors initially focused on genes showing enhanced expression at sites of cardiovascular lesion, and especially aimed to isolate genes with locally enhanced expression in the heart by using the subtraction method (see Swaroop *et al.*, Nucleic Acids Res. 19: 1954 (1991)). As a result, a novel gene of approximately 12-kb in full length, whose expression is increased in the heart following the administration of the L-NAME NO synthase inhibitor was isolated by screening a cDNA library. The novel gene obtained was named the LACS (L-NAME-related actin cytoskeletal protein) gene. Northern blot analysis showed that this gene is expressed in the heart and skeletal muscles. A particularly strong mRNA expression was confirmed in myocardial cells of the heart. Cellular distribution showed co-localization and expression with some of the actin stress fibers. Immunoprecipitation analysis showed that the expressed protein binds (directly or indirectly) to actin fibers, and Western blotting also showed it to be in the skeletal fraction. Furthermore, the amino acid sequence predicted from the nucleotide sequence of this gene was analyzed for its functions, properties, and such, and no characteristic sequences including signal sequences and transmembrane regions were found. However, a proline-rich sequence was present in the C terminus, and this sequence was found to be homologous to an SH3-binding domain.

The above-mentioned results, along with the large size of the gene and so on, indicated that LACS is a structural protein related to the cytoskeleton. LACS mRNA was abundantly expressed in a blood-pressure independent manner in the hearts of several model animals with hypertension and cardiomegaly (L-NAME rats, AngII infusion rats, and spontaneously hypertensive rats (SHRs)). In cultured myocardial cells, increased LACS mRNA expression due to hypertrophic agonist stimulation was observed. Furthermore, the expression mechanism was suggested to involve the AngII-AT1R pathway and the Rho/ROCK system. LACS was thought to increase in expression along actin upon hypertrophic stimuli, bind directly or indirectly to actin, and participate in the reorganization of actin fibers through functional modulation of actin. The above-mentioned increase of mRNA expression by angiotensin (AngII), phenylephrine, endothelin-1, and such suggests that at least a portion of LACS expression is regulated by the downstream signaling of the G-protein-coupled receptor.

This gene, which is highly expressed in hypertension and cardiomegaly model animals, has increased expression in cultured myocardial cells due to hypertrophic agonist

stimulation, and encodes a protein that was suggested to associate with the modulation of actin polymerization, is expected to be used as a pharmaceutical for cardiac diseases such as cardiac failure, cardiomegaly, myocarditis, cardiomyopathy, arteriosclerosis, arteriosclerosis obliterans, or ischemic heart disease.

Accordingly, the present invention provides:

(1) A protein selected from (a) to (d):

(a) a protein comprising the amino acid sequence of SEQ ID NO: 1;

(b) a protein comprising the amino acid sequence of SEQ ID NO: 1, wherein one or more amino acids have been modified by deletion, substitution, addition, and/or insertion;

(c) a protein comprising a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions with a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 2; and

(d) a protein comprising an amino acid sequence having 60% homology to the amino acid sequence of SEQ ID NO: 1;

(2) A polynucleotide encoding the protein of claim 1 or a portion thereof;

(3) The polynucleotide of claim 2, which comprises the nucleotide sequence of SEQ ID NO: 2;

(4) A pharmaceutical comprising the protein of claim 1;

(5) The pharmaceutical of claim 4, which is used to prevent, improve, or treat cardiac failure, cardiomegaly, myocarditis, cardiomyopathy, arteriosclerosis, arteriosclerosis obliterans, or ischemic heart disease;

(6) A pharmaceutical comprising the polynucleotide of claim 2; and

(7) The pharmaceutical of claim 6, which is used to prevent, improve, or treat cardiac failure, cardiomegaly, myocarditis, cardiomyopathy, arteriosclerosis, arteriosclerosis obliterans, or ischemic heart disease.

The present invention provides the novel gene LACS, which plays an important role in cardiovascular lesions. The nucleotide sequence of the LACS cDNA is shown in SEQ ID NO: 2, the amino acid sequence of the LACS protein predicted from the nucleotide sequence and encoded by the LACS cDNA is shown in SEQ ID NO: 1.

The proteins of this invention are proteins comprising the amino acid sequence of SEQ ID NO: 1. These proteins can be obtained, for example, from cells producing these proteins using an affinity chromatography column to which antibodies against the protein are bound. The proteins can also be purified by conventional protein purification techniques based on the molecular weight of the LACS protein (373 kDa) and their binding affinity to actin. Since the expression of LACS protein in cultured myocardial cells can be induced by

L-NAME administration, L-NAME-induced LACS protein can be isolated and purified by: salting out; chromatography such as gel filtration chromatography, ion-exchange chromatography, reverse-phase chromatography, affinity chromatography, hydrophobic chromatography, adsorption chromatography, and such; gel electrophoresis; ultrafiltration; re-crystallization; distillation; dialysis; isoelectric focusing; filtration; immunoprecipitation; solvent extraction; solvent precipitation; and such (see, ed. Marshak *et al.*, Strategies for Protein Purification and Characterization: A Laboratory Course Manual, Cold Spring Harbor Press (1996)).

Fusion proteins are included in the proteins comprising the amino acid sequence of SEQ ID NO: 1. Examples of such fusion proteins are: the proteins of this invention to which a signal sequence instructing host cells to secrete is added for easy purification, when the proteins are expressed by genetic engineering techniques; and those attached with a tag comprising FLAG, histidine residues, or such for easy recovery, or a tag such as GFP for detection. Fusion proteins cleavable by thrombin, Xa factor, or such using conventional techniques can be prepared, and portions other than the portions corresponding to the proteins of this invention can be deleted as necessary.

The proteins of this invention can also be proteins comprising an amino acid sequence obtained by modifying the amino acid sequence of SEQ ID NO: 1, through deletion, substitution, addition, and insertion of one or more amino acids. Such proteins can be obtained by modifying and expressing the polynucleotides encoding proteins comprising the amino acid sequence of SEQ ID NO: 1 by commonly used genetic techniques. Genetic modification techniques include, for example, the site-directed mutagenesis method (ed. Ausubel *et al.*, Current Protocols in Molecular Biology, publish. John Wiley & Sons, section 8.1-8.5 (1987)). Such modified proteins can also be used to prepare fusion proteins as described above, as necessary.

Furthermore, the proteins of this invention are proteins comprising a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions with a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 2. Such proteins can be obtained by, for example, preparing probes based on the nucleotide sequence of SEQ ID NO: 2, screening a mammalian cDNA library, genomic library, and such by the hybridization method (ed. Ausubel *et al.*, Current Protocols in Molecular Biology, publish. John Wiley & Sons, section 6.3-6.4 (1987)), and expressing the obtained polynucleotides. However, the phrase "polynucleotide that hybridizes under stringent conditions with the polynucleotide comprising the nucleotide sequence of SEQ ID NO: 2" as used in this invention is not intended to restrict the method for producing such polynucleotides to those obtained by the hybridization method. Therefore, polynucleotides that can be produced by techniques such as the aforementioned

site-directed mutagenesis are included in the definition, as long as they hybridize under stringent conditions with the nucleotide sequence of SEQ ID NO: 2. Herein, the term "stringent conditions" refers to conditions of low salt concentration or high temperature in the washing step, and includes conditions of 1x SSC, 0.1% SDS, 37°C (or 55°C).

The proteins of this invention include proteins comprising an amino acid sequence homology of 50% or more, preferably 70% or more, more preferably 80% or more, even more preferably 90% or more, and most preferably 95% or more (for example, 96%, 97%, 98%, 99%, or more) to the amino acid sequence of SEQ ID NO: 1. Such proteins can be obtained by the above-mentioned site-directed mutagenesis and hybridization method, PCR method (ed. Ausubel *et al.*, Current Protocols in Molecular Biology, publish. John Wiley & Sons, section 6.1-6.4 (1987)), and such. The homologies in this invention are determined using the BLAST algorithm (Karlin and Altschul, Proc. Natl. Acad. Sci. USA 90: 5873-7 (1993)). Programs such as BLASTX (Altschul *et al.*, J. Mol. Biol. 215: 403-10 (1990)) that have been developed based on the BLAST algorithm are known. One can refer to "http://www.ncbi.nlm.nih.gov." for the specific analytical procedures.

The LACS protein of this invention is highly expressed in the hearts of several hypertension and cardiomegaly model animals, and is encoded by a gene whose expression increases locally in the heart upon administration of the L-NAME NO synthase inhibitor. Therefore, the protein expression can be used as a basis for detecting the inhibition of NOS expression or activity, and enables the diagnosis of diseases induced by decreased NOS expression or activity. Detection of the protein expression is not limited thereto, and can be done using antibodies against the protein. An Antibody against a protein of this invention can be produced by conventional techniques using apparently the protein comprising the amino acid sequence of SEQ ID NO: 1, or a portion thereof, or using an above-mentioned protein of this invention that has the antigenicity of the protein comprising the amino acid sequence of SEQ ID NO: 1. Therefore, the proteins of this invention and peptide fragments thereof can be used to produce antibodies that enable the diagnosis of diseases induced by decreased NOS expression or activity. When the proteins of this invention are used as pharmaceuticals for humans, human-derived LACS protein is preferably used, but is not limited thereto. Human-derived LACS protein can be obtained by generating probes or primers based on the sequence information of the LACS protein and gene of this invention, obtaining a gene encoding the desired protein using the above-mentioned hybridization method or various PCR methods, and expressing this gene.

The proteins of this invention include proteins that are functionally equivalent to the proteins comprising the amino acid sequence of SEQ ID NO: 1. Herein, the phrase "functionally equivalent to the proteins comprising the amino acid sequence of SEQ ID NO:

1" refers to having the activity to enhance or suppress the polymerization, crosslinking, or bundle formation of actin. Proteins functionally equivalent to the LACS protein comprising the amino acid sequence of SEQ ID NO: 1 also include proteins that can be obtained by using the above-mentioned site-directed mutagenesis method, hybridization method, PCR method, and such.

Furthermore, the present invention provides polynucleotides that encode the proteins of this invention, or portions thereof. Such polynucleotides include cDNAs, genomic DNAs, mRNAs, and chemically synthesized DNAs and RNAs. A single protein of the present invention can be encoded by a plurality of polynucleotides due to the degeneracy of the genetic code. Thus, the polynucleotides of this invention also include such degenerate polynucleotides. Naturally occurring polynucleotides of this invention can be obtained by, for example, generating probes, primers, and such based on the entire or a portion of the nucleotide sequence encoding the LACS protein of this invention comprising SEQ ID NO: 2, and performing well-known techniques such as hybridization and PCR. Furthermore, if necessary, the obtained polynucleotides can be modified by, for example, restriction enzyme digestion, site-directed mutagenesis, or addition of a suitable fragment (including linkers, initiation codons and stop codons), linked to a polynucleotide encoding a different polypeptide for fusion protein expression, or integrated into an appropriate vector (expression vectors, cloning vectors, and such). The polynucleotides of this invention also include these polynucleotides.

The present invention also provides polynucleotides comprising at least 13 consecutive nucleotides complementary to the nucleotide sequence of SEQ ID NO: 2 or its complementary strand. Such complementary polynucleotides do not have to be completely complementary to the nucleotide sequence of SEQ ID NO: 2 or its complementary strand, as long as they have homologies of at least 70% or higher, preferably 80% or higher, more preferably 90% or higher, and even more preferably 95% or higher. Homology can be determined according to the aforementioned methods. Such polynucleotides can be used as probes or primers for the detection and amplification of DNAs and mRNAs encoding the proteins of this invention. When the polynucleotides are used as primers, restriction enzyme recognition sequences and/or tags, for example, can be added to their 5' ends as necessary. The polynucleotides may also be used as antisense nucleotides, ribozymes, and such. Antisense nucleotides and ribozymes can be used for inhibiting or suppressing the expression of the proteins of this invention.

The proteins of this invention can be obtained by integrating the polynucleotides of this invention into appropriate expression vectors under the control of an expression regulatory region comprising enhancers, promoters, and such, and introducing the vectors into

appropriate host cells. Examples of the host cells are prokaryotic cells and eukaryotic cells. For eukaryotic cells, systems using *E. coli* are well known. Examples of the promoters used when the host is *E. coli* are lacZ promoter (Ward *et al.*, Nature 341: 544-6 (1998)), and ara promoter (Better *et al.*, Science 240: 1041-3 (1988)). When *E. coli* is used as a host to produce the proteins of this invention by genetic engineering methods, attachment of a signal sequence that enables the production of proteins into the periplasm is desired for easy purification. An example of the signal sequence is pelB signal sequence (Lei *et al.*, J. Bacteriol. 169: 4379 (1987)). The expression vectors for *E. coli* may further include the replication origins derived from SV40, polyomavirus, adenovirus, bovine papilomavirus, and such, and selection markers such as aminoglycoside transferase gene, thymidine kinase gene, xanthine guanine phosphoribosyl transferase gene, and dihydrofolate reductase gene. In addition to *E. coli*, prokaryotic expression systems using *Bacillus subtilis* as the host are well known.

For eukaryotic hosts, yeast cell systems, plant cell systems, and animal cell systems including insect cells, amphibian cells, and mammalian cells are known. Generally used yeast cell systems include systems that use a *Saccharomyces* yeast or an *Aspergillus* mold as host. For plant cell systems, those that use *Nicotiana tabacum* cells as hosts are known. In plant cell systems, proteins can be produced in callus cultures, or obtained by regenerating plants from cells transfected with the desired gene and obtaining the proteins of interest from the leaves, roots, stems, and such of the plants (Julian *et al.*, Eur. J. Immunol. 23: 131-8 (1994)). Examples of the mammalian cells include BHK, CHO, COS, HeLa, myeloma, and 3T3. Xenopus oocytes (Valle *et al.*, Nature 291: 358-40 (1981)) are known as amphibian cells while Sf9, Sf21, Tn5, and such are well-known insect cells. The constructed vectors are introduced into host cells by well-known methods such as the calcium phosphate method (Virology 52: 456-67 (1973)), and electroporation method (EMBO J. 1: 841-5 (1982)).

The proteins of this invention can also be produced *in vivo* using animals (see Lubon, Biotechnol. Annu. Rev. 4: 1-54 (1998)). Examples of the animals are: domestic animals such as cattle, sheep, pigs, and goats (Ebert *et al.*, Bio/Technology 12: 699-702 (1994)); mammals such as mice; insects such as silkworms. For the production of an exogenous protein in mammals, a DNA encoding the protein of interest is fused with a gene encoding a protein specifically secreted in milk, such as β-casein. Next, the fusion gene is injected into the embryo of the animal to induce chromosomal recombination. The protein of interest can be obtained from the milk produced by the transgenic animals (i.e., those born as a result of transplanting this embryo transplanted into the uterus of a female animal) or from their offspring. Alternatively, when silkworms are used, baculovirus that carries an integrated gene encoding the desired protein is used to infect silkworms, and the desired protein can be

obtained from the silkworm body fluids (Susumu *et al.*, Nature 315: 592-4 (1985)).

Proteins that have been secreted inside or from host cells as a result of genetic engineering can be purified by the same method as for the naturally occurring proteins. For proteins that have been optionally modified for the convenience of purification, the modified peptide portions can be removed by reacting with an appropriate protein modification enzyme before or after protein purification.

Antibodies against the proteins of this invention can be obtained using the proteins of this invention, or portions thereof. The antibodies in this description include polyclonal antibodies, monoclonal antibodies (Milstein *et al.*, Nature 305: 537-40 (1983)), and antibody fragments. A polyclonal antibody against a protein of this invention may be, for example, serum obtained from the blood of a mammal sensitized with an antigenic portion of a protein of this invention. This serum can be further purified to prepare fractions comprising the polyclonal antibody. On the other hand, monoclonal antibodies can be prepared using the hybridoma method (Kohler and Milstein, Nature 256: 495 (1975)), by collecting immunocytes from mammals sensitized with an antigen; cloning hybridomas by fusing the collected cells with cells capable of permanent proliferation, such as myeloma cells; collecting monoclonal antibodies from the culture.

Antibody fragments refer to fragments comprising the antigen-binding region or variable region of an antibody, and include Fab, Fab', F(ab')2, and Fv fragments and such. Fab is a fragment obtained by papain digestion of antibody molecules. Pepsin digestion of an antibody yields the F(ab')2 fragment. As for other antibody fragments, examples include diabodies (Holliner *et al.*, Proc. Natl. Acad. Sci. USA 90: 6444-8 (1993)), filamentous antibodies, single chain antibodies such as scFV (Plucktun, "The Pharmacology of Monoclonal Antibody", Vol. 113, ed. Rosenburg and Moore, Springer Verlag, pp.269-315 (1994)), and multispecific antibodies (LeDoussal *et al.*, Int. J. Cancer Suppl. 7: 58-62 (1992); Paulus, Behring Inst. Mitt. 78: 118-32 (1985); Millstein and Cuello, Nature 305: 537-9 (1983); Zimmermann, Rev. Physiol. Biochem. Pharmacol. 105: 176-260 (1986); Van Dijk *et al.*, Int. J. Cancer 43: 944-9 (1989)). Furthermore, the antibodies of this invention can be modified by molecules such as polyethylene glycol. The antibodies thus obtained can be purified by methods similar to those for purifying other proteins (ed. Harlow and David Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory (1988)).

Since the proteins of this invention are expressed specifically in myocardial cells, and increase in diseased conditions such as left ventricular hypertrophy, they may be used to prevent, improve, or treat cardiac diseases such as cardiac failure, cardiomegaly, myocarditis, cardiomyopathy, arteriosclerosis, arteriosclerosis obliterans, or ischemic heart disease. Therefore, by enhancing or regulating the functions of the proteins of this invention, these

proteins can be formulated as agents to prevent, improve, or treat these cardiac diseases. When the proteins are used as pharmaceuticals, they can be administered to a patient directly and on their own, or formulated using conventional formulation methods. Examples of such methods include dissolving into a neutral solution such as PBS. Furthermore, pharmaceutically acceptable stabilizers, buffers, sweeteners, diluents, corrigents, fillers, coloring agents, emulsifiers, excipients, disintegrating agents, flavoring agents, preservatives, solubilizers, and such may be added as necessary. By combining with carriers, the proteins of this invention can be prepared into forms such as solutions, elixirs, capsules, granules, pills, suspensions, powders, tablets, syrup, injections, troches, and emulsions.

The dosage of the pharmaceuticals comprising the proteins of this invention depends on a number of factors such as the weight, age, and symptoms of the patient, as well as the method and form of administration. One skilled in the art can determine the appropriate dosage. The pharmaceuticals can be, for example, administered subcutaneously or orally, or administered by intraarterial or intravenous injection. The daily protein dosage for adults (body weight of 60 kg) is normally 1 μg to 10 g, preferably 10 μg to 1 g, and more preferably 100 μg to 100 mg. Furthermore, the dosage can be calculated based on the body weight when administering into animals other than humans.

Instead of the proteins of this invention, polynucleotides encoding these proteins may be used as pharmaceuticals. Similarly to pharmaceuticals comprising the proteins of this invention, pharmaceuticals comprising such a gene as an active ingredient can be used to prevent, improve, or treat cardiac diseases such as cardiac failure, cardiomegaly, myocarditis, cardiomyopathy, arteriosclerosis, arteriosclerosis obliterans, or ischemic heart disease. Hereinafter, methods, forms, and amounts of gene transfer will be described specifically for gene therapies using the polynucleotides of this invention.

Methods for administering gene therapy agents comprising a LACS gene as an active ingredient can be classified into two groups: methods using non-viral vectors and methods using viral vectors. The preparation methods, administration methods, and such for these vectors are described in detail in experiment manuals (Jikken Igaku (Experimental Medicine) Supplementary Volume, "Idenshichiryo no Kisogijyutsu (Fundamental Techniques for Gene Therapy)", Yodosha, 1996; Jikken Igaku (Experimental Medicine) Supplementary Volume, "Idenshidonyu & Hatsugenkaiseki Jikkenho (Experimental Methods for Gene Transfer & Expression Analysis)", Yodosha, 1997; "Idenshi-chiryo Kaihatsu Kenkyu Handbook (Handbook of Gene Therapy Research and Development)", Nihon Idenshichiryo Gakkai (The Japan Society of Gene Therapy) Edition, NTS, 1999).

The target gene can be introduced into cells or tissues by using the methods below and a recombinant vector prepared by inserting a target gene into a conventional non-viral

gene expression vector. Examples of methods for transferring genes into cells are: lipofection methods, calcium-phosphate co-precipitation methods, DEAE-dextran methods, methods that directly infuse DNA using a glass capillary tube, etc. Methods for transferring genes into tissues include methods using virus envelope vectors, internal type liposomes, electrostatic type liposomes, HVJ-liposomes, improved type HVJ-liposomes (HVJ-AVE liposomes), receptor-mediated gene transfer methods, methods for transferring carriers (such as metal particles) along with DNAs using particle guns, methods for directly introducing naked-DNAs, introduction methods using positively charged polymers, etc.

The aforementioned HVJ-liposomes are constructed by incorporating a DNA into a liposome formed by a lipid bilayer, then fusing this liposome with an inactivated Sendai virus (hemagglutinating virus of Japan; HVJ). The use of HVJ-liposomes is characterized by extremely high cell membrane fusion compared to conventional liposome methods, and is one of the especially preferred forms of introduction. Methods for preparing HVJ-liposomes are described in detail in, for example, Experimental Medicine Supplementary Volume, "Idenshichiryo no Kisogijyutsu (Fundamental Techniques of Gene Therapy)", Yodosha, 1996; Experimental Medicine Supplementary Volume, "Idenshidonyu & Hatsugenkaiseki Jikkenho (Experimental Methods for Gene Transfer & Expression Analysis)", Yodosha (1997); J. Clin. Invest. 93: 1458-64 (1994); Am. J. Physiol. 271: R1212-20 (1996). Methods for using the HVJ-liposome are described in, for example, Molecular Medicine 30: 1440-8 (1993); Jikken Igaku (Experimental Medicine), 12: 1822-6 (1994); and Tanpakushitsu Kakusan Kouso (Protein, Nucleic Acid, and Enzyme), 42: 1806-13 (1997); and a more preferable method is described in Circulation 92 (Suppl. II): 479-82 (1995).

Furthermore, methods using viral envelopes are particularly preferable when administering a LACS gene of this invention. Viral envelopes can be prepared by mixing a purified virus with an expression vector of interest in the presence of a surfactant, or by freezing and thawing a mixture of a virus and an expression vector (JP-A 2001-286282).

The viruses that can be used in the viral envelope methods are viruses belonging to families such as the retrovirus, togavirus, coronavirus, flavivirus, paramyxovirus, orthomyxovirus, bunyavirus, rhabdovirus, poxvirus, herpesvirus, baculovirus, and hepadnavirus families, and HVJs are particularly preferable. Herein, these viruses can be either wild-type or recombinant viruses. In particular, a recombinant HVJ reported by Hasan, M. K. *et al.* (J. General Virol. 78: 2813-20 (1997)), Yonemitsu, Y. *et al.* (Nature Biotech. 18: 970-3 (2000)), or such may be used as an HVJ.

While the Z strain (available from ATCC) of HVJ is generally preferable in methods using HVJ-liposomes or HVJ-envelopes, fundamentally, other HVJ strains (for example, ATCC VR-907 and ATCC VR-105) can also be used. When preparing a viral envelope,

purified viruses can be inactivated by UV irradiation and such, and mixed with a desired expression vector. Surfactants that can be used for mixing the virus and expression vector include, for example, octylglucoside, Triton X-100, CHAPS, and NP-40. Viral envelope vectors prepared in this manner can be introduced by injection or such into tissues to be targeted for therapy, prevention, or remedy. Furthermore, by freezing at -20°C the viral envelope vectors can be stored for at least two to three months.

Any expression vector may be used here as long as they can express a target gene *in vivo*. Examples of the expression vectors are pCAGGS (Gene 108: 193-200 (1991)), pBK-CMV, pcDNA3.1 (Invitrogen), and pZeoSV (Stratagene).

Representative methods for gene transfer with viral vectors are those methods using viral vectors such as recombinant adenoviruses and retroviruses. More specifically, a target gene can be transferred into cells by the steps of: introducing the gene into DNA or RNA viruses such as detoxicated retroviruses, adenoviruses, adeno-associated viruses, herpesviruses, lentiviruses, vaccinia viruses, poxviruses, polioviruses, sindbis viruses, Sendai viruses, SV40, or human immunodeficiency viruses (HIV) (see Pharmacol. Ther. 80: 35-47 (1998); Front. Biosci. 4: E26-33 (1999); J. Recep. Signal. Transduct. Res. 19: 673-86); and then infecting cells with the resultant recombinant virus. The infection efficiency of adenovirus vectors is much greater than the other aforementioned viral vectors. Thus, from this viewpoint, the use of an adenovirus vector system is preferred.

Methods for introducing an agent of the present invention to a patient during gene therapy include: the *in vivo* introduction of a gene therapy agent directly into the body; and the *ex vivo* introduction of a gene therapy agent into a cell harvested from the patient, followed by reintroduction of the modified cell into the body (Nikkei Science, April 1994, 20-45; Gekkann Yakuji 36 (1), 23-48, 1994; Jikken Igaku (Experimental Medicine) Supplementary Volume, 12 (15), 1994; "Idenshi-chiryo Kaihatsu Kenkyu Handbook (Handbook of Gene Therapy Research and Development)", Nihon Idenshichiryo Gakkai eds. (The Japan Society of Gene Therapy) Edition, NTS, 1999). *In vivo* methods are particularly preferred in the present invention.

Various formulations, (for example, liquid preparations), suited for each of the above-mentioned administration methods may be adopted as the form of preparation. For example, an injection comprising a gene as an active ingredient can be prepared by conventional methods, which might include dissolving a gene in an appropriate solvent (e.g., a buffer solution, such as PBS, physiological saline, and sterilized water), sterilizing by filtration as necessary, and then loading into a sterile container. Conventional carriers or such may be added to injection agents as required. Alternatively, liposome preparations, such as preparations comprising HVJ-liposome, can be prepared as suspensions, frozen agents, or

centrifugally concentrated frozen agents.

The dosage of the pharmaceuticals comprising the polynucleotides of this invention depends on a number of factors such as the weight, age, and symptoms of the patients, as well as on the method and form of administration. One skilled in the art can determine the appropriate dose. The pharmaceuticals can be, for example, administered subcutaneously or orally, or by intraarterial or intravenous injection. The daily polynucleotide dosage for adults (body weight of 60 kg) is normally 1 μg to 10 g, preferably 10 μg to 1 g, and more preferably 100 μg to 100 mg. Furthermore, the dosage can be calculated based on the body weight when administering to animals other than humans.

More specifically, since the polynucleotides of this invention can be repeatedly administered when using the HVJ-envelope method, the gene is administered a number of times, for example, twice or three times, but not all together at once, in order to obtain better therapeutic, preventive, or improvement effects. The present invention also includes such types of administration which are performed over a number of times using the HVJ envelope.

The appropriate administration methods and sites to be administered for the pharmaceuticals comprising the proteins or polynucleotides of this invention are selected according to the disease and symptoms to be treated. The preferred administration method is intramyocardial or intramuscular administration, but is not limited thereto.

Since the LACS gene of this invention was found to increase in the heart of several hypertension and cardiomegaly model animals, it can be used to diagnose the presence or absence of hypertension and cardiomegaly in patients. Such diagnoses may be performed by detecting intracellular mRNAs transcribed from the gene, using probes or primers generated based on the sequence information of the LACS gene of this invention. Extraction of mRNAs from biological samples can be also performed using a commercially available kit (such as the mRNA Purification Kit (Pharmacia) and QuickPrep mRNA Purification Kit (Pharmacia)). In addition, methods for preparing total RNAs, such as guanidine ultracentrifugation methods (Chirgwin *et al.*, Biochemistry 18: 5294-9 (1979)) and AGPC methods (Chomczynski and Sacchi, Anal. Biochem. 162: 156-9 (1987)), are well known. Meanwhile, methods for detecting proteins expressed from these genes using antibodies against the proteins of this invention may also be considered. When examining the indicator gene expression in cells, its expression level is usually corrected with the measured expression levels of genes whose expression levels do not vary substantially with the cellular condition (housekeeping genes such as β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are often used).

The LACS protein and the LACS gene of this invention can be used to screen for compounds useful as pharmaceuticals for cardiovascular diseases. As indicated in the

present invention, *in vivo* expression of the LACS protein increases in response to the administration of NO synthetic inhibitors or hypertrophic agonists. Therefore, compounds that bind to the LACS protein may be candidates of pharmaceuticals for cardiovascular diseases. Such compounds can be screened, for example, by the steps of:

(1) contacting a test compound with a proteins of this invention or a partial peptide thereof;

(2) detecting the binding of the test compound to the protein, or partial peptide; and

(3) selecting a test compound that binds to the protein, or the partial peptide.

Alternatively, binding of test compounds to proteins of this invention can be investigated by contacting test compounds with host cells that maintain the expression of polynucleotides encoding the proteins of this invention, or with a host cell culture, instead of the proteins of this invention or partial peptides thereof in step (1).

In the present invention, the LACS protein was shown to bind to actin. By inhibiting the binding between the LACS protein and actin, actin polymerization may be enhanced or suppressed. Therefore, compounds that inhibit the binding between the LACS protein and actin may be candidates of pharmaceuticals for cardiovascular diseases. Methods for screening compounds that inhibit the binding of the LACS protein can be performed using the binding between the protein and actin as an index. More specifically, such compounds can be screened, for example, by the steps of:

(1) contacting a test compound with a protein of this invention or a partial peptide thereof in the presence of actin;

(2) detecting the binding of actin to the protein or partial peptide; and

(3) selecting a test compound that suppresses or inhibits the binding of actin to the protein or partial peptide.

The partial peptides used here must comprise the portion(s) involved in the binding between the LACS protein and actin. Such partial peptides can be obtained easily by analyzing the actin affinity of the various fragments produced upon digestion of the LACS protein. Furthermore, binding between the LACS protein and actin may be carried out by methods as described in Example 7, using LACS antibodies and actin antibodies, but is not limited thereto.

The present invention also provides methods of screening for compounds that regulate the expression of the proteins of this invention. Compounds that enhance or suppress the expression of the LACS protein may be candidates of pharmaceuticals for cardiovascular diseases. Such screening can be performed using myocardial cells and smooth muscle cells expressing the LACS gene by the steps of:

(1) contacting a test compound with cells expressing the LACS gene;

(2) detecting the expression of LACS gene; and

(3) selecting a test compound that enhances or suppresses the expression of the LACS gene compared to when the test compound is absent.

Various cells including *E. coli*, yeast, insect cells, plant cells, oocytes, and mammalian cells can be used for the expression of the LACS gene. Similarly to the detection of the *in vivo* expression of LACS gene in patients, transcribed mRNAs and proteins can be detected according to conventional methods.

Furthermore, cells that have been transformed with an expression vector, in which a reporter gene is operably linked to an expression regulatory sequence upstream of the LACS gene, can be used in place of cells that express the LACS gene. Examples of the upstream expression regulatory sequence of the LACS gene are promoters, enhancers, CAAT box, and TATA box. For example, probes are generated based on the nucleotide sequence of SEQ ID NO: 2, and genomic DNA clones comprising the expression regulatory sequence of the LACS gene is obtained by screening a genomic DNA library. The expression regulatory sequence portion of this clone is excised by restriction enzyme treatment, and then cloned to be operably linked to the upstream of an appropriate reporter gene. Examples of the reporter gene are chloramphenicol acetyltransferase (CAT) gene, lacZ gene, luciferase gene, green fluorescent protein (GFP) gene, and growth hormone gene. The obtained construct in which a reporter gene is linked downstream of the LACS gene expression regulatory sequence is introduced into an appropriate host cell (preferably mammalian cells) by any one of the above-described methods. When this construct is intended for the insertion into the host cell chromosome, homologous recombination techniques may be used.

Expression of the reporter gene is detected by methods appropriate for the type of reporter gene employed. For example, the level of CAT gene expression is determined by the detection of chloramphenicol acetylation due to the gene expression product. When using the lacZ gene, the coloring catalysis of the coloring compound in the gene expression product is measured as an indicator of the expression level of the gene. When the reporter gene is the luciferase gene, fluorescence of a fluorescent compound that results from the catalysis of the gene expression product is detected and this can be used as an indicator of expression level. Since GFP protein emits fluorescence, when the GFP gene is used as the reporter gene, the expression level is determined according to the fluorescence level of the expression product. When using the growth hormone gene, the effects (growth stimulation, proliferative stimulation, and such) of the gene expression product on cells are examined and the expression level is quantified.

Examples of test compounds to be used in each of the above-mentioned screening methods include naturally occurring compounds, synthetic compounds, inorganic compounds, organic compounds, and proteins, peptides, and non-peptide compounds that are crude,

purified or partially purified. In addition, compound libraries comprising a plurality of compounds, expression products derived from a gene library, cell extracts, cell culture supernatants, products of luminescent microorganisms, extracts of marine organisms, plant extracts, biological tissue extracts, and such may also be used. Proteins or peptides can be used as test compounds by binding with a carrier, fusing with another polypeptide, or expressing on a cell membrane and preparing membrane fractions. These test compounds can be labeled with radiolabeling, fluorescence labeling, and such, as necessary.

Cells, expression vectors, test compounds, probes, primers, antibodies, substrates for measuring reporter gene expression, and such, which are necessary for each of the above-mentioned screening methods, can be appropriately combined into kits. Furthermore, the kits may include, as necessary, media and containers for cell culturing, control samples, kit instructions, and such. Test compounds selected by the above-mentioned screening can be made into pharmaceuticals to be used directly on patients by themselves, but they may also be formulated and used according to conventional formulation methods, as necessary. When the compounds are polynucleotides such as DNAs, or polypeptides that can be encoded by DNAs, the polynucleotides may be administered according to the aforementioned gene therapeutic methods.

Best Mode for Carrying Out the Invention

Hereinafter, the present invention will be specifically described using Examples, but it is not to be construed as being limited thereto.

[Example 1] Gene isolation by Subtractive Hybridization (SSH)

RNAs were extracted from the hearts of a male WKY rat group that has been subjected to a one-week oral administration of L-NAME (100 mg/L), and from the hearts of a control group without L-NAME administration. The obtained RNAs were used to clone cDNAs that showed enhanced expression in the L-NAME-administered group using the subtraction method. As a result, a novel LACS (L-NAME related actin cytoskeletal protein) gene was successfully isolated. Northern blotting was used to analyze the mRNA expression of this gene in cardiac tissues, and showed that the expression level reaches its peak one to three days after L-NAME administration, begins declining from the seventh day, and gradually decreases to nearly the baseline 28 days after administration. Furthermore, mRNA expression in tissues other than the heart was also analyzed by Northern blotting. The LACS mRNA expression was observed in the heart and skeletal muscles. Particularly with the heart, the expression of LACS mRNA was confirmed in the myocardial cells.

[Example 2] Isolation of full-length cDNA

A cDNA library was constructed, and a full-length cDNA encoding LACS was isolated by screening. More specifically, poly RNAs obtained from WKY rats on the first day of L-NAME administration were used to construct a λZAPII cDNA library using random primers. Next, by repeated screening using the LACS gene fragments as probes, an approximately 12-kb cDNA in full length was obtained as the LACS gene. Sequencing of this cDNA was performed using the ABI PRISM310 DNA Sequencer (ABI/ Perkin Elmer). The obtained nucleotide sequence is shown in SEQ ID NO: 1. Characteristic sequences such as signal sequences or transmembrane regions could not be found in the amino acid sequence (SEQ ID NO: 2) predicted from the nucleotide sequence. Therefore, it was difficult to predict the properties and functions of LACS from the sequence alone. However, a proline-rich sequence exists at the C terminus of the predicted amino acid sequence, suggesting an SH3-binding domain homology. SH3 is a homologous portion of approximately 70 amino acids seen in the Src kinase family. The SH3-binding domain is a proline-rich sequence of approximately ten amino acids.

[Example 3] mRNA expression in the hearts of hypertension models

Cardiac tissues derived from AngII infusion rats (osmotic pump: 0.7 mg/kg/day), and SHRs of 4 weeks and 24 weeks, were used. AT1R antagonist (ARB) and hydrazine (Hyd) were used as antihypertensive agents. Each of the antihypertensive agents was used at an amount sufficient for lowering the blood pressure (10 mg/kg/day for ARB; 12 mg/kg/day for Hyd).

The results present a trend of increased LACS expression after the third day of AngII administration, and decreased expression after the seventh day in the AngII infusion rats. Most of this increase in expression tended to be suppressed by a simultaneous ARB administration, but not by the simultaneous administration of hydrazine. However in SHRs, a significant increase of LACS mRNA expression was observed in adult rats that had developed hypertension with progressing cardiomegaly, but not in juvenile rats that had not yet developed hypertension.

As described above, increase of LACS mRNA expression was observed in the hearts of several hypertension and cardiomegaly model animals, and even the correction of hypertension did not completely suppress this expression. These findings suggest the possibility that the LACS mRNA expression is amplified by local activation of the RAS system. Therefore, cellular response to AngII stimulation was examined in Example 6.

[Example 4] Localization of the LACS protein

The C-terminal portion of LACS was used to prepare an antibody against LACS. Intracellular localization of LACS was examined by cellular staining using the peptide antibody.

(1) Localization in the heart

LACS was stained in the cardiac tissue in a band pattern, which appeared to be consistent with the intercalated discs. Double staining with cadherin, a representative protein that exists in the intercalated discs, and observation using a confocal fluorescence microscope showed that LACS localization virtually matched with that of cadherin. Thus, LACS seemed to exist near the intercalated discs.

(2) Localization in cultured myocardial cells

Primary cultures of myocardial cells isolated from neonatal rats (one- to three-day old) using trypsin and collagenase were prepared. For the first 24 to 48 hours, the cells were cultured in a serum-containing medium, and then for another 24 hours in a serum-free medium.

Western blot analysis of the cultured myocardial cells showed that the LACS protein was fractionated into the cytoskeletal component (TritonX insoluble fraction). In immunostaining, staining along the actin fibers, particularly near the sites of intercellular adhesion, was observed. The sites of intercellular adhesion are said to have an *in vivo* intercalated disc-like structure, and thus LACS was considered to exist along the actin fibers at the sites of intercellular adhesion.

These results showed that LACS is a cytoskeletal protein.

[Example 5] LACS expression

A c-myc tag was attached to LACS, and this construct was inserted into expression vectors, pcDNA3.1 (Invitrogen) and pEGFP (CLONTECH). The obtained expression vectors were transfected into COS-1 cells using FuGene6 (Roche Diagnostics) for transient overexpression. After 48 hours, an approximately 374-kDa band, corresponding to the molecular weight predicted theoretically from the LACS nucleotide sequence, was detected by Western blotting against c-myc. Furthermore, the expression of c-myc-fused LACS protein at the cellular level was confirmed by c-myc immunostaining after immobilizing the cells,.

[Example 6] LACS expression increases due to hypertrophic agonist stimulation on cultured myocardial cells

Myocardial cells cultured by the same method in Example 4 were used. First, cells

were stimulated with the representative hypertrophic agonists, AngII, phenylephrine, and Endothelin-1. LAC protein expression was detected in each of the cultured cells, and was found to increase when any of the hypertrophic agonists was used.

LACS expression which increases upon phenylephrine stimulation was suppressed by the simultaneous administration of 10 μM of Y27632 (ROCK inhibitor; Calbiochem). This suggests that the Rho/ROCK system is related to the expression of LACS gene. Rho is a low-molecular-weight GTP-binding protein (small G protein) involved in the contraction of smooth muscles, and regulation of cytokinesis, cell motility, and cytomorphology, via reorganization of actin filaments.

[Example 7] Detection of actin

Immunoprecipitation was performed using the LACS antibody to obtain precipitated proteins from cultured myocardial cells. A band corresponding to the actin protein was detected by Western blotting using an anti-actin antibody.

This result shows that LACS and actin bind to each other. Immunostaining in Example 4 suggests the possibility of LACS interacting with cadherin since its localization matches that of cadherin. However, a band corresponding to cadherin was not detected in the above-mentioned experiment, and therefore the possibility of direct binding to cadherin was rejected.

[Example 8] LACS expression in the carotid arteries

Analysis of LACS mRNA expression by RT-PCR confirmed its expression in the carotid arteries. After balloon injury, mRNA expression gradually increased and peaked on the seventh day. This suggests the possibility that LACS exists in the smooth muscles. Since smooth muscles are stable and readily cultured, they are convenient for further functional analyses of LACS.

Industrial Applicability

The present invention provides novel actin-related cytoskeletal protein LACS and genes encoding this protein. The present invention reveals that: the expression of the LACS protein is increased in the heart of several hypertension and cardiomegaly model animals; this expression increases when hypertrophic agonists are administered; and the LACS protein is bound to actin in cells. These facts suggest that the LACS protein plays a specific role in the maintenance of the cardiovascular system by enhancing or suppressing actin polymerization. Therefore, the proteins of this invention and polynucleotides encoding these proteins may be effective for the prevention, improvement, or treatment of cardiac diseases involving actin

polymerization, such as cardiac failure, cardiomegaly, myocarditis, cardiomyopathy, arteriosclerosis, arteriosclerosis obliterans, and ischemic heart disease.